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**Keys words**: multiple sclerosis, clinically isolated syndrome, MRI, USPIO, macrophage

# Ultra-small Super Paramagnetic Iron Oxide predicts higher disease activity in clinically isolated syndrome

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**Running title**: USPIO in clinically isolated syndrome

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## Abstract

**Background**: Macrophages are important components of inflammatory processes in multiple sclerosis, closely linked to axonal loss, and can now be observed *in-vivo* using Ultra-Small super-Paramagnetic Iron Oxide (USPIO). We aimed to determine the prevalence of macrophage infiltration and to assess the predictive value on disease activity and tissue injury after one year in clinical isolated syndrome patients.

**Methods**: Thirty-five patients were imaged using conventional-MRI, magnetization transfer ratio (MTR) to assess tissue destructuration, gadolinium (Gd) to probe blood brain barrier integrity, and USPIO to study macrophage infiltration.

**Results**: At baseline, patients showed 17 USPIO-positive lesions reflecting infiltration of macrophages present from the onset. This infiltration was associated with higher local tissue destructuration as emphasized by lower MTR values of USPIO-positive/Gd-positive lesions compared to USPIO-negative/Gd-positive and to non-enhanced lesions, at baseline and Month-12, and no difference between USPIO-negative/Gd-positive and non-enhanced lesions. While at baseline T2-lesion load of patients with USPIO-enhancement compared to patients with Gd-enhancement was not different, it was higher at Month-12. T1-lesion load was also higher at Month-12 in patients with USPIO-enhancement.

**Conclusion**: Infiltration of activated macrophages evidenced by USPIO enhancement, is present at the onset of MS and is associated with higher local and global progression of tissue destructuration.

## Introduction

Macrophage infiltration is an important component of the inflammatory processes associated with multiple sclerosis. Several studies support a close relationship between macrophage infiltration and axonal loss. First, colocation of active macrophages and axonal injury has been reported [1],[2]. Secondly, macrophages synthesize free radicals and cytotoxic proteins [3] known to cause axonal loss [4],[5], through mitochondrial injury and subsequent energy failure [6]. Furthermore, axonal injury is a major substrate for permanent neurological disability in patients [7],[8]. Currently, the magnetic resonance imaging (MRI) marker of inflammation used in clinical routine is the T1-signal enhancement produced by gadolinium chelate-based contrast agents. Gadolinium chelates passively cross the damaged blood brain barrier, diffusing into the intercellular space. While gadolinium contrast agents are largely used in daily practice and allow the depiction of active inflammatory lesions [9], they are non-specific and indirect markers of inflammatory cell infiltration in multiple sclerosis. Recently, new contrast agents based on particles of ultra-small super paramagnetic iron oxide (USPIO) have been proposed as new candidates for brain macrophage infiltration imaging. Phagocytosis of USPIO by monocytes/macrophages cells allows the *in vivo* and non-invasive labeling of regions with macrophage infiltration [10]. USPIO causes local decreases in longitudinal and transversal relaxation times leading to MRI contrast changes. This labeling has been shown to be specific to monocytes/macrophages in animal models [11],[12] and in

humans [13]. Preliminary studies have proven the feasibility of using USPIO in multiple sclerosis patients [13]–[16]. These four studies have all initially focused on radiological pattern descriptions and spatial distributions of USPIO enhancements in relapsing-remitting multiple sclerosis (RRMS) and progressive multiple sclerosis patients. They have shown that USPIO provides distinct and complementary information to gadolinium-enhanced MRI. These studies have also suggested an association between USPIO enhancement patterns [13],[15] and subsequent regional macroscopic tissue destructuration demonstrated by the occurrence of chronic black holes.

In patients at the first stage of the disease, the potential existence and prognostic value of USPIO enhancement has never been investigated. One may suppose that in clinically isolated syndrome (CIS) suggestive of multiple sclerosis, USPIO enhancement may be present and predict higher disease activity. To test this hypothesis, we performed a one-year multi-centre longitudinal study in CIS patients using USPIO contrast imaging and multi-modal MRI.

## Materials and Methods

### Patients and Study Design

Thirty-five patients (13 males, 22 females) were included in a prospective longitudinal study within three months after a first demyelinated clinical episode suggestive of multiple sclerosis. Data from thirty-one patients were analyzed at baseline and at month-12 (M12) as four patients did not show up at M12. Patients were included after screening in five different French University hospitals (Rennes, Marseille, Paris, Toulouse, Reims) based on the following criteria: (i) age between 18 and 45; (ii) occurrence of the first presumed inflammatory demyelinating event in the central nervous system involving either the optic nerve, the spinal cord, a brain hemisphere, or the brainstem; (iii) no previous history of neurological symptoms suggestive of demyelination; (iv) no possible alternative diagnoses (lupus erythematous, antiphospholipid antibody syndrome, Behcet disease, sarcoidosis, Lyme's disease, cerebral arteritis, brain lymphoma, etc.); (v) patients fulfilling at least the dissemination in space criteria according to Polman et al 2005 [17]; (vi) EDSS (Expanded Disability Status Scale) between 0 and 5 at baseline; (vii) first infusion of USPIO within three months after the first clinical episode; (viii) no corticoids in the month before USPIO infusion and no previous administration of immunomodulatory or immunosuppressive drugs; (ix) no previous history of asthma, allergy, infusion of iron oxide particles within 5 months; (x) no pregnancy.

The local ethics committees approved the protocol and all subjects gave their informed written consent.

Patients' disability was rated using the Kurtzke Expanded Disability Status Scale (EDSS) at baseline and M12, on the day of the MRI exam.

## Image acquisition

Patients were scanned with 3T commercially available MRI systems (Verio MR system Siemens, Erlangen Germany in Marseille and Rennes and Achieva MR system Philips, Amsterdam, Netherlands in Reims) at baseline and 12-months later (M12).

Conventional and quantitative MRI were acquired at baseline in two steps: before (day 1) and 24 hours after USPIO infusion (day 2). The first day, before USPIO infusion, the protocol included transverse fast spin-echo proton density-weighted and T2-weighted sequences (Verio: $TR/TE_1/TE_2$ = 6530/8.8/88ms and Achieva: $TR/TE_1/TE_2$ = 2269/8.2/90ms; all other parameters were the same: 44 contiguous sections, 3-mm section thickness, in-plane resolution 1mmx1mm), 2D gradient-echo $T2^*$-weighted sequences (TR/TE = 50/27ms, 44 contiguous sections, 3-mm section thickness, Verio: in-plane resolution 1.3mmx1.3mm and Achieva: 1mmx1mm), transverse proton density-weighted spoiled gradient-echo sequences (Verio: TR/TE=750/4.5ms and Achieva: TR/TE=65.8/5.1ms, all other parameters: 44 contiguous sections, 3-mm section thickness, in-plane resolution 1mmx1mm) performed without ($M_0$) and with

($M_{mt}$) magnetization transfer (MT) saturation (Gaussian shape, 1.5-kHz off-water resonance, 500° for Verio , 620° for Achieva). Transverse spin-echo T1-weighted sequence (Verio: TR/TE = 500/8.4 ms and Achieva: TR/TE = 600/9.3ms; all other parameters: 44 contiguous sections, 3-mm section thickness, in-plane resolution 1mmx1mm) was also performed before and five minutes after intravenous administration of 0.1 mmol/kg of gadolinium (Gd) chelate (gadopentetate dimeglumine, Magnevist®, Bayer Schering Pharma, Berlin-Wedding) to identify lesions enhanced by Gd. After the MR exam, USPIO (SHU-555C; Bayer Schering Pharma, Berlin-Wedding) was injected over approximately 30 minutes (40 μmol of iron/kg of body weight).

The second day (24 hours after USPIO infusion), the transverse spin-echo T1-weighted sequence was performed to identify lesions enhanced by USPIO.

For each patient, a follow-up MR exam was performed at M12 using the same protocol except for the infusion of USPIO.

## Safety

Patients were monitored clinically after USPIO infusion and every three months. All data were collected in case report forms.

## Image analysis

All conventional images were blindly analyzed by three experts (JCF, IB, AT). The visual analysis consisted of post gadolinium and post USPIO T1-enhanced lesion count and pattern of enhancement analysis according to three classes: ring-like enhancement, focal enhancement and return to iso-intensity of a pre-contrast hypo-intense lesion [13].

The overlap of USPIO and gadolinium enhancement was rated using a 3-point scale: <20%, 20-80% and >80% of the volume.

T2 lesions were delineated at baseline and M12 on the T2-weighted images. Persistent T1 hypointense lesions (so-called chronic black holes) were delineated at M12 onto the T1-weighted images by means of a semi-automated method [18] by the same experienced neurologist (AM).

Magnetization Transfer Ratio (MTR) maps were calculated on a voxel-by-voxel basis according to the following equation: $MTR = ((M_0 - M_{mt})/M_0)$, where $M_0$ and $M_{mt}$ were the images obtained respectively without and with the magnetization transfer saturation pulse.

T2-weighted images were coregistered onto the $M_{mt}$ images using the normalized mutual information procedure (SPM5, Welcome institute, London). The coregistered mask of the T2 lesions was subtracted from the $M_{mt}$ images to obtain the lesion-free $M_{mt}$ images. Voxels of the coregistered T2 lesion mask were set at a value

corresponding to the mean voxel values of the normal-appearing white matter (NAWM) and subsequently added to the lesion-free $M_{mt}$ image. This in-painting procedure yielded to "normal-like" $M_{mt}$ images (devoid of lesions) for each patient, and prevented the misclassification of lesions into gray matter (GM) during the segmentation procedure. Then, segmentation of these images into GM and white matter (WM) maps was performed (SPM5). Finally, the GM and WM probability masks thresholded at 75% and the T2 lesion masks were applied to the MTR maps. At the end of the pipeline, we obtained MTR values of each lesion, classified according to the enhancement type, and MTR values of NAWM and GM of each patient [19].

## Statistical analysis

Patients were classified according to the type of lesion enhancement: patients with at least one USPIO enhanced lesion (UEL-group), patients with only Gadolinium enhanced lesions (GEL-group), and patients with no enhanced lesions (NEL-group).

Three-groups comparisons between UEL-group, GEL-group and NEL-group were performed to compare age, EDSS, T2 and T1 lesion loads, NAWM MTR, GM MTR using the Kruskall Wallis test corrected for paired comparisons with the Steel-Dwass procedure ($p<0.05$). Comparison of genders was assessed using the Fisher exact test ($p<0.05$).

Radiological evolution of lesions T1-w intensity (iso or hypointense in T1-w images) was assessed using Fisher exact test ($p<0.05$).

Considering lesions, comparisons of MTR values of lesions depending on the type of enhancement ($USPIO^{+}/Gd^{+}$; $USPIO^{-}/Gd^{+}$ and $USPIO^{-}/Gd^{-}$) were assessed using Kruskall Wallis test corrected for paired comparisons with the Steel-Dwass procedure ($p<0.05$).

The software used for this statistical assessment was JMP 9.0.0, SAS Institute Inc.

## Results

### Demographic and clinical characteristics of patients (Table 1)

Considering the 31 patients followed in the study, their mean age at baseline was 32.1 (±SD=8.3) years. The mean period between the first inflammatory demyelinating event and the first MR exam was 66.3 (±21.7) days. No patients received any treatment at the onset. No patients developed any adverse events. All patients converted to multiple sclerosis at M12 according to 2005 McDonald criteria [17].

Patients were classified into 3 groups according to their enhancement at baseline: (i) the UEL-group for patients showing at least one USPIO enhancing lesion and at least one Gd enhancing lesion (n=9); (ii) the GEL-group for patients showing lesions enhanced only by Gd (n=7); and (iii) the NEL-group for patients showing no enhanced lesions (n=15).

There was no difference between these three groups in terms of age, sex, mean disease duration and baseline EDSS (Table 1).

### Prevalence of USPIO and Gd enhancement (Figure 1)

USPIO enhancement was hyperintense on T1-w images whereas no signal changes were observed in these lesions on T2*-w images.

In the whole group of 31 patients, 16 $USPIO^{+}/Gd^{+}$ enhanced lesions, 67 $USPIO^{-}/Gd^{+}$ enhanced lesions and 643 $USPIO^{-}/Gd^{-}$ non-enhanced lesions were depicted. Only one

lesion was $USPIO^+/Gd^-$ positive and converted to $USPIO^-/Gd^+$ at M12. According to this marginal pattern, this lesion was not taken into account for the statistical analysis.

Among the 17 USPIO positive lesions (16 $USPIO^+/Gd^+$ and 1 $USPIO^+/Gd^-$) observed at baseline, three different patterns of USPIO enhancement were characterized: pattern 1 was lesions with ring-like enhancement (n=4), pattern 2 was lesions with focal enhancement (n=3) and pattern 3 was lesions returning to isointensity after USPIO infusion compared to the pre-contrast hypo-intense signal of the lesion (n=10) (Figure 1).

For the 16 $USPIO^+/Gd^+$ enhanced lesions, overlaps of USPIO and Gd enhancements were less than 20% for 3 lesions and between 20% and 80% for 13 lesions. No overlap greater than 80% was observed.

The enhanced lesions (n=83) at baseline (16 $USPIO^+/Gd^+$ and 67 $USPIO^-/Gd^+$) did not show any enhancement at M12.

**Relationships between regional USPIO/Gd enhancement and lesional tissue structure assessed by MTR (Figure 2)**

At baseline, considering the 31 patients, the mean MTR values of lesions were significantly lower in the $USPIO^+/Gd^+$ lesions (n=16; $MTR_{USPIO+/Gd+}$=0.38±0.05) relative to the $USPIO^-/Gd^+$ lesions (n=67; $MTR_{USPIO-/Gd+}$=0.42±0.04; p=0.03) and to the

USPIO$^-$/Gd$^-$ lesions (n=643; $MTR_{USPIO-/Gd-}$=0.41±0.06; p=0.04). No difference was found in MTR between the USPIO$^-$/Gd$^+$ lesions and the USPIO$^-$/Gd$^-$ (p=0.93).

At M12, no enhancement was observed but the mean MTR values were significantly lower in the lesions with the USPIO$^+$/Gd$^+$ baseline pattern (n=16; $MTR_{USPIO+/Gd+}$=0.40±0.05) relative to lesions with USPIO$^-$/Gd$^+$ baseline pattern (n=67; $MTR_{USPIO-/Gd+}$=0.43±0.05; p=0.03), and no significant difference was found in MTR of lesions with the USPIO$^+$/Gd$^+$ baseline pattern relative to lesions with the USPIO$^-$/Gd$^-$ baseline pattern (n=643; $MTR_{USPIO-/Gd-}$=0.42±0.06; p=0.08). No difference was found in the MTR values between lesions with USPIO$^-$/Gd$^+$ baseline pattern and lesions with USPIO$^-$/Gd$^-$ baseline pattern (p=0.28).

### T1-intensity according to USPIO/Gd lesion enhancement status

Eight out of the sixteen (50%) USPIO$^+$/Gd$^+$ lesions at baseline evolved to chronic black holes at M12 while forty-seven out of the sixty-seven (70%) USPIO$^-$/Gd$^+$ lesions at baseline evolved to chronic black holes at M12 (p=0.11). Focusing on the pattern of USPIO enhancement, the subgroup of lesions returning to isointensity pattern was more associated with transiently T1-hypointense lesions (70%) compared to the two other patterns (16%) (p=0.06). Furthermore, we noticed that, in most of the cases, persistent T1-hypointensity at M12 was colocalized with the USPIO area enhancement at baseline for the ring-like and the focal enhancement patterns.

## T1 and T2 lesion load according to USPIO/Gd enhancement status in patients

At M12, total T1 lesion load was significantly higher in the UEL-group of patients (8.5±10.2 $cm^3$) compared to the GEL-group (2.5±1.9 $cm^3$; p=0.04) and to the NEL-group (2.5±3.8 $cm^3$; p=0.02). No difference was found between patients in the GEL and NEL groups (p=0.43).

Concerning total T2 lesion load, at baseline, a significant difference was found between the UEL-group of patients (13.0±20.8 $cm^3$) compared to the NEL-group (3.1±4.4 $cm^3$; p=0.02) while no significant difference was found with the GEL-group (4.9±3.2 $cm^3$; p=0.68) neither between the GEL-group and the NEL-group (p=0.21).

At M12, total T2 lesion load was significantly higher in the UEL-group (11.4±10.9 $cm^3$) compared to the GEL-group (3.8±2.2 $cm^3$; p=0.03) and to the NEL-group (3.6±4.5 $cm^3$; p<0.01) while no difference was found between the GEL-group and the NEL-group (p=0.52).

## MTR values in normal appearing white matter and gray matter according to USPIO/Gd enhancement status in patients

There was no significant difference between the three groups of patients in terms of the mean MTR value inside the NAWM at baseline ($MTR_{NAWM\text{-}UEL}$= 0.46±0.05; $MTR_{NAWM\text{-}GEL}$= 0.47±0.04 and $MTR_{NAWM\text{-}NEL}$= 0.46±0.05; p=0.88) and at M12

($MTR_{NAWM\text{-}UEL}$= 0.46±0.05; $MTR_{NAWM\text{-}GEL}$= 0.47±0.05 and $MTR_{NAWM\text{-}NEL}$= 0.47±0.05; p=0.35). The same pattern was observed in the GM at baseline ($MTR_{GM\text{-}UEL}$= 0.38±0.06; $MTR_{GM\text{-}GEL}$= 0.39±0.05 and $MTR_{GM\text{-}NEL}$= 0.37±0.06; p=0.86) and at M12 ($MTR_{GM\text{-}UEL}$= 0.37±0.06; $MTR_{GM\text{-}GEL}$= 0.39±0.06 and $MTR_{GM\text{-}NEL}$= 0.37±0.06; p=0.75).

**Disability level according to USPIO/Gd enhancement status in patients**

At baseline, mean EDSS in the whole group of patients was 1.17±0.81. No significant difference was found between the EDSS of the three groups of patients at baseline ($EDSS_{UEL\text{-}group}$= 1.33±0.87; $EDSS_{GEL\text{-}group}$= 0.85±0.90 and $EDSS_{NEL\text{-}group}$= 1.32±0.70; p=0.46).

At M12, mean EDSS was 1.08±1 for all patients. No significant difference was found between the EDSS of the three groups of patients at M12 ($EDSS_{UEL\text{-}group}$= 1.05±1.05); $EDSS_{GEL\text{-}group}$= 1.5±1.05 and $EDSS_{NEL\text{-}group}$= 0.92±0.93; p=0.52).

## Discussion

This study demonstrates that infiltration of activated macrophages, highlighted by USPIO enhancement, is already present at the onset of multiple sclerosis and is associated with higher progression of tissue destructuration.

USPIO enhancement reflects active migration of macrophages across the blood brain barrier after USPIO phagocytosis in peripheral circulation, especially in the lymph nodes and spleen [13],[20],[21]. The present study evidenced, for the first time, an infiltration of activated macrophages in clinically isolated syndrome (CIS). The proportion of USPIO positive lesions among the inflammatory active lesions appears lower compared to the previous studies performed in more advanced stages of the disease (RRMS) [13]–[15]. This finding could be related to a lower macrophage activity in CIS compared to RRMS. In addition, whatever the stage of the disease, the delay between the last clinical attack and USPIO infusion may influence the proportion of USPIO positive lesions explaining the higher level of USPIO lesions in the previous studies that included only patients during an active phase of the disease [13]–[15].

We found that USPIO uptake was mainly depicted at the periphery of multiple sclerosis lesions and never completely overlapped gadolinium-enhanced areas. The pattern of USPIO enhancements may reflect the presence of active macrophages known to be particularly located at the periphery of acute multiple sclerosis lesions [22]. Therefore,

the pattern of USPIO enhancement underlies the high specificity of this contrast agent to reveal macrophage infiltration.

In this study, the presence of at least one USPIO positive lesion at baseline in patients is associated with a higher T2 lesion load during the one year following period. Considering that level of T2 lesions accumulation during the first years of multiple sclerosis has already been demonstrated as the best prognostic marker of poor disease outcome [23]–[25], we may suppose that patients showing USPIO enhancement will suffer from higher long-term disability. In addition, we demonstrated that the group of patients with at least one USPIO positive lesion also showed higher T1 lesion load accumulation during the follow-up. In the literature, the level of irreversible T1 lesions accumulation was associated with long-term disability and seems to better explain disability worsening than T2 lesion accumulation [26]. Therefore, our study emphasizes that USPIO enhancement at the onset of multiple sclerosis appears to predict a less favorable outcome at long-term. This finding has to be confirmed with a longer follow up study.

A main finding of our study is that tissue injury assessed by MTR imaging was more severe in lesions enhanced by USPIO compared to all other lesions. This difference remained present one year later. This result is consistent with a previous study performed in an animal model showing a MTR decrease in USPIO enhanced lesions [27]. In humans, the existence of a relationship between USPIO enhancement and

higher tissue damage was reported in more advanced multiple sclerosis patients using the count of T1 hypointensity commonly used as a measure of severe tissue damage [15]. The pathophysiological processes leading to more severe tissue injury in lesions marked by USPIO remain unknown. In particular it is not clear if macrophage infiltration contributes directly to tissue destructuration or only reflects the involvement of these inflammatory cells in the phagocytosis of myelin and cellular fragments.

In conclusion, macrophage infiltration is present from the earliest stage of the disease and is associated with a major and persistent tissue destructuration and an unfavorable outcome at medium term. The potential predictive value of USPIO enhancement in term of future disability will be validated in further long-term studies.

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## Table and Figures legends

**Table 1**: **Demographic and clinical characteristics of patients.**

"UEL-group" for patients showing at least one USPIO enhancing lesion; "GEL-group" for patients showing lesions enhanced only by gadolinium; "NEL-group" for patients showing no enhanced lesions. M12= month-12 follow-up. EDSS = Expanded Disability Status Scale.

**Figure 1. Radiological patterns of USPIO enhancement.**

(A) Ring-like enhancement; (B) Focal enhancement and (C) Return to iso-intensity of a pre-contrast hypointense lesion

**Figure 2. MTR values at baseline and M12 according to the lesion type.**

Significant differences are represented by (*)

| | **All patients** (n=31) | **UEL-group** (n=9) | **GEL-group** (n=7) | **NEL-group** (n=15) |
|---|---|---|---|---|
| **Age** (years) Mean (±SD) | 32 ±8 | 31 ±9 | 33 ±9 | 32 ±8 |
| **Gender** | 22 F/ 9M | 5F / 4M | 6F / 1M | 11F/ 4M |
| **Delay 1st event – 1st MRI** (days) Mean (±SD) | 66 ±22 | 60 ±21 | 69 ±32 | 69 ±16 |
| **Treatment at baseline** | none | none | none | none |
| **EDSS at baseline** Mean (±SD) | 1.17 ±0.81 | 1.33 ±0.87 | 0.85 ±0.90 | 1.32 ±0.70 |
| **EDSS at M12** Mean (±SD) | 1.08 ±1 | 1.05 ±1.05 | 1.5 ±1.05 | 0.92 ±0.93 |
| **Conversion to MS at M12** | 100% | 100% | 100% | 100% |
| **$T_2$ Lesions Load at baseline** ($cm^3$) Mean (±SD) | 6.5 ±12.2 | 13.0 ±20.8 | 4.9 ±3.2 | 3.1±4.4 |
| **$T_2$ Lesions Load at M12** ($cm^3$) Mean (±SD) | 6.0 ±7.4 | 11.4 ±10.9 | 3.8 ±2.2 | 3.6 ±4.5 |
| **$T_1$ Lesions Load** at M12 ($cm^3$) Mean (±SD) | 4.3 ±6.6 | 8.5 ±10.2 | 2.5 ±1.9 | 2.5 ±3.8 |

**Table 1**: **Demographic and clinical characteristics of patients.**
"UEL-group" for patients showing at least one USPIO enhancing lesion; "GEL-group" for patients showing lesions enhanced only by gadolinium; "NEL-group" for patients showing no enhanced lesions. M12= month-12 follow-up. EDSS = Expanded Disability Status Scale.

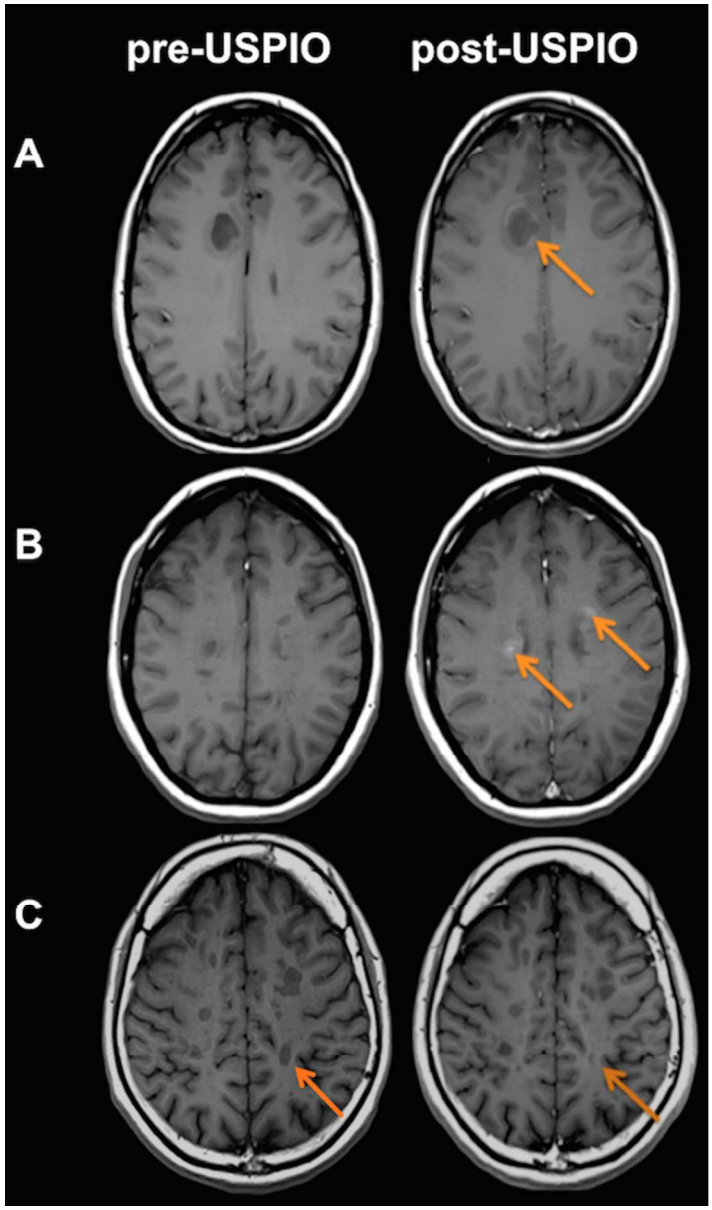


Figure 1. Radiological patterns of USPIO enhancement.
(A) Ring-like enhancement; (B) Focal enhancement and (C) Return to iso-intensity of a pre-contrast hypointense lesion
90x153mm (300 x 300 DPI)

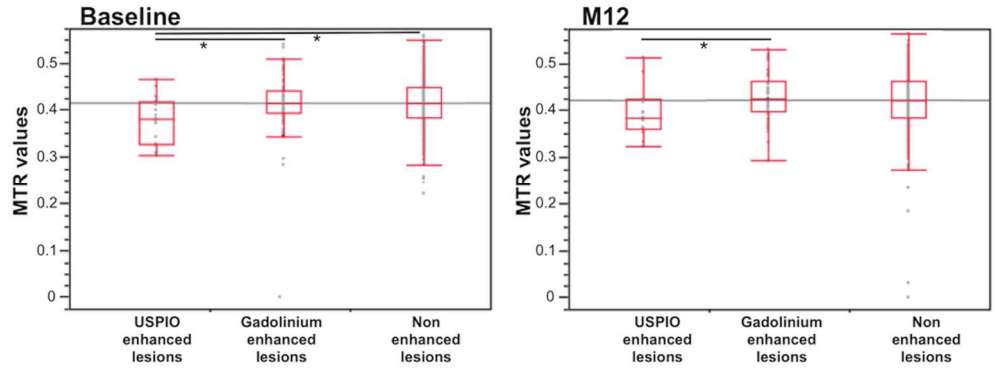


Figure 2. MTR values at baseline and M12 according to the lesion type.
Significant differences are represented by (*)
184x69mm (300 x 300 DPI)